NITROGEN FIXATION IN ERICACEAE

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(WITH FOUR FIGURES)

Introductory

Since the middle of the nineteenth century it has been known that plants belonging to the Ericaceae form mycorhiza of a characteristic kind. Further knowledge of the relations between plant and endophyte in this group has only recently been forthcoming.

In 1915 RAYNER[1] showed that the relationship in *Calluna vulgaris* is of a remarkable character, involving obligate symbiosis between the two organisms and a much more extensive distribution of the fungus throughout the green plant than had been suspected. As in Orchidaceae, root formation by seedlings is dependent upon early infection by the endophyte, failing which, development ceases and the plant perishes in the seedling stage. Unlike the condition in Orchidaceae, infection at the appropriate moment is provided for by the presence of mycelium on the seed coat, a condition ensured by the distribution of the endophyte throughout the vegetative tissues and eventually within the ovary chamber. These facts have been demonstrated with certainty in *Calluna*, and the evidence points to a similar condition throughout the family. Thus ovarial infection has been reported for many species in all the suborders of Ericaceae, and the inability of seedlings to complete their development without infection has already been proved for a number of these.

In such remarkable associations between flowering plants and fungi as are found in the orchids and in Ericaceae, it is of great interest to learn the exact nutritive relations between the symbionts. In orchids there is ocular evidence of digestion of mycelium by the cells of the root, and it is clear that by this means the plant can draw indirectly upon organic compounds of carbon and nitrogen in the soil. In the chlorophyllous orchids the endophyte can utilize

[1] RAYNER, M. C., Obligate symbiosis in *Calluna vulgaris*. Ann. Botany **29**:97–153. 1915.

the products of photosynthesis, but in the nonchlorophyllous forms, such as *Neottia* and *Corallorhiza*, this is not so, and, on the observed facts, the mutual relationship appears to be one of parasitism on the part of the green plant. Indeed this condition has fully been demonstrated for *Gastrodia elata*, a remarkable non-chlorophyllous species found in Japan. It is certain, therefore, that one at least of the so-called "saprophytic" orchids is parasitic upon a fungus, *Armillaria mellea*, and that the establishment of this relation has become obligate for the full development of the plant. This is the more interesting in that the fungus concerned is parasitic in habit and invades the tuber of the orchid in the first instance in exactly the same manner as it attacks the tubers of potato, upon which it is commonly found as a parasite in Japan.[2] In orchids the fungi endophytic in the roots do not spread into the chlorophyllous tissues, nor is there any evidence that they can use atmospheric nitrogen.

In *Calluna* the evidence as to exchange of food materials between the two partners may be summarized as follows. There is no indication of digestion of mycelium by the root, nor are there any obvious symptoms of attack or defense beyond the fact that hyphae effect an entry in the first instance and spread from cell to cell. That this vegetative activity depends upon a supply of food drawn from the plant cells rather than from organic compounds in the soil, is suggested by the normal behavior of the symbionts when grown in solutions of inorganic salts in pure culture. In the shoot, active mycelium is not readily demonstrated although widely distributed in a reduced condition; active hyphae occur in the extensive air spaces of the leaves, and grow into the air from the surface of the shoot. Moreover, there is evidence that mycelium undergoes digestion by the mesophyll cells of the leaf, and also that the fungus can hydrolyze arbutin outside the plant (RAYNER, *loc. cit.*). With respect to nitrogen assimilation, there is cumulative evidence that the endophyte of Ericaceae can utilize atmospheric nitrogen in greater or less degree, and it is the purpose of this paper to present this as briefly as possible.

[2] KUSANO, S., *Gastrodia elata* and its symbiotic association with *Armillaria mellea*. Jour. Coll. Agric. Tokyo 4:1–66. 1911.

The experimental evidence in question is derived from three sources. (1) The work of TERNETZ,[3] who showed that certain fungi isolated from the roots of ericaceous species could utilize atmospheric nitrogen. (2) The work of the writer (*loc. cit.*), which supplied the necessary link connecting these fungi directly with Ericaceae, and also provided additional evidence of the ability of certain ericaceous species to utilize atmospheric nitrogen. (3) The work of DUGGAR and DAVIS,[4] who undertook a critical experimental review of the difficult problem of nitrogen fixation by fungi. The evidence provided by these workers will now be considered in historical sequence.

(1) The researches of TERNETZ were undertaken in connection with an attempt to isolate the root endophytes of Ericaceae, concerning which no information was at that time available. As a result of prolonged experiments, eight pycnidia-forming fungi were isolated, five of which were investigated for evidence of fixation of gaseous nitrogen. All forms isolated were referred by LINDAU and HEMMINGS to *Phoma*, and differed in the small size of the pycnidiospores (4–5 μ in length) from the species previously found associated with Ericaceae. The five forms experimented with were isolated from the roots of *Oxycoccus palustris*, *Andromeda polifolia*, *Vaccinium Vitis-Idaea*, *Erica Tetralix*, and *E. carnea*, and were named *Phoma radiciis Oxycocci*, *P. radiciis Andromedae*, *P. radiciis Vaccini*, *P. radiciis Tetralicis*, and *P. radiciis Ericae*, respectively. TERNETZ has put on record the interesting observation that these fungi, although isolated from plant species growing in close proximity, are specific strains, distinguishable by definite morphological and physiological characters.

The isolation of fungal species endophytic in the roots of plants is a matter of notorious difficulty, and their identity can only be proved by formation of mycorhiza typical for the species following upon inoculation from pure culture into the roots of seedlings free from fungal infection. Those isolated by TERNETZ were

[3] TERNETZ, C., Über die Assimilation des atmosphärischen Stickstoffes durch Pilze. Jahrb. Wiss. Bot. 44:353–408. 1907.

[4] DUGGAR, B. M., and DAVIS, A. R., Studies in the physiology of the fungi. I. Nitrogen fixation. Ann. Mo. Bot. Gard. 3:413–437. 1916.

believed to be the endophytes associated with the different species, but the necessary proof was lacking, inasmuch as seedlings of the latter were never obtained free from mycorhiza. All attempts to sterilize seeds failed, since sooner or later the roots of seedlings raised from such seeds showed the mycorhizal condition typical of the species in nature. The five strains of *Phoma*, as well as *Asper-*

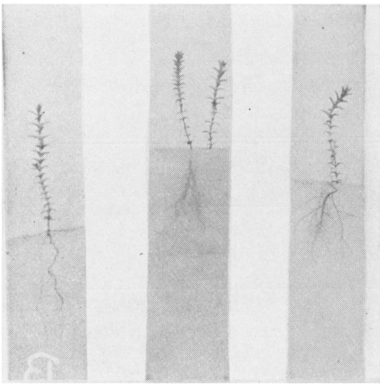

FIG. 1

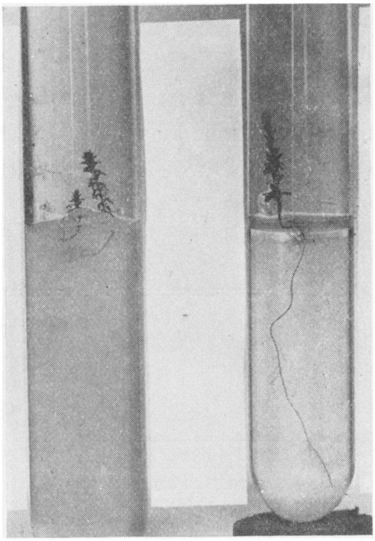

FIG. 2

FIG. 1, 2.—Fig. 1, *Calluna vulgaris:* representative seedlings (four months old) from large number grown in agar nutrient lacking nitrates; growth continued until root system occupied whole of rooting medium; shoots averaged 3.5–5 cm. length; fig 2, *Calluna vulgaris:* representative seedlings (three months old) from silica jelly cultures lacking combined nitrogen; silica nutrient in right-hand tube chanced to be more liquid in consistency than other tube, or than tubes shown in fig. 3, hence more vigorous root development; finer roots in upper part of root system not visible in photograph.

gillus niger and *Penicillium glaucum*, were subsequently cultivated on media free from combined nitrogen. The cultures were carried on over a period of several years, were frequently repeated, and due precautions were observed with regard to purity of materials, adequacy of controls, and methods of estimation. It is recorded that none of the fungi investigated required a supply of combined nitrogen for healthy development or growth. They all fixed atmospheric nitrogen, but in very different degrees. The values

obtained for *Aspergillus* and *Penicillium* are too small to have any critical value, and agree in this respect with those of previous investigators. The highest capacity for nitrogen fixation was found in the strains of *Phoma* isolated from *Oxycoccus*, *Vaccinium*, and *Andromeda*, in which the values cited appear to be well outside the range of any possible experimental error. Table I, reproduced from the original paper by TERNETZ, shows the values obtained for *Phoma radiciis* as compared with those on record for the nitrogen-fixing bacteria.

TABLE I

RECORDS OF NITROGEN FIXATION BY FUNGI EXTRACTED FROM FOUR ERICACEOUS SPECIES, AS COMPARED WITH THAT EXHIBITED BY *Clostridium*, *Azotobacter* *Aspergillus*, AND *Penicillum*, FROM TERNETZ (*loc. cit.*).

ORGANISM	DAYS	DEXTROSE SUPPLIED		DEXTROSE USED (GM.)	NITROGEN FIXATION		INVESTIGATOR
		gm.	Percentage		mg.	per gm. dextrose (mg.)	
Clostridium Pasteurianum..	20	40	4	40	53.6	1.34	Winogradski
Clostridium Pasteurianum..	20	20	2	20	24.4	1.22	Winogradski
Clostridium americanum...	30	1.25	0.25	1.25	4.6	3.7	Pringsheim
Clostridium americanum...	30	5	1	3.01	8.2	3.01	Gerlach and Vogel
Azotobacter chroococcus...	35	5	0.5	5	42.7	8.56	Gerlach and Vogel
Azotobacter chroococcus...	35	12	1.2	12	127.9	10.66	Ternetz
Aspergillus niger..........	28	7	7	1.1	1.9	1.71	Ternetz
Penicillium glaucum.......	28	7	7	0.7	2.8	3.8	Ternetz
Phoma radiciis Oxycocci...	28	7	7	0.85	15.3	18.08	Ternetz
Phoma radiciis Andromedae	28	7	7	0.67	7.3	10.92	Ternetz
Phoma radiciis Vaccinii....	28	7	7	0.71	15.7	22.14	Ternetz
Phoma radiciis Tetralicis...	28	7	7	1	4	3.99	Ternetz
Phoma radiciis Ericae......	28	7	7	1.1	2.3	2.17	Ternetz

The three fungal strains concerned work much less energetically but more economically than *Clostridium* or *Azotobacter*. For example, for each gram of dextrose used, 22 mg., 18 mg., and 11 mg. of nitrogen were combined, as compared with values ranging from 1.2 mg. to 10.6 mg. of nitrogen per gram of dextrose for the nitrogen-fixing bacteria. These are the highest relative figures on record for nitrogen-fixing organisms.

(2) The evidence contributed by the writer was obtained in the course of an intensive experimental study of *Calluna vulgaris*, and supplied the link needed to connect the fungi isolated by TERNETZ

with their ericaceous host plants. In ignorance of the work of TERNETZ, the conclusion was reached independently that seedling pots become infected from the testa subsequent to germination. This view proved to be correct, and a pycnidia-bearing fungus was eventually isolated with comparative ease from unopened fruits. Proof of the identity of this fungus was then provided by reinoculation into seedlings grown in pure culture and raised from sterilized seeds. A remarkable condition of obligate symbiosis was thus put on record for *Calluna*, and the observations made by TERNETZ as to the specificity of the fungal strains in the different ericaceous species were subsequently confirmed. The characters of the endophyte isolated from *Calluna* agree with those described by TERNETZ, and the necessary proof is thus provided that the forms experimented with by this worker were actually those associated with the five plant species concerned. In view of this fact, the suggestion previously put forward as to nomenclature (*loc. cit.*, p. 125) should be withdrawn and the name *Phoma radiciis Callunae* accepted.

In the paper recording these facts, attention was drawn to observations bearing on the possibility of nitrogen fixation by the endophyte. Of these may be mentioned: (1) the vigor and longevity of seedlings germinated on filter paper moistened with distilled water (RAYNER[5]); (2) the wide distribution of the endophyte throughout the plant tissues, its development in the intercellular spaces of the leaves and emergence to the air from the surface of the shoot; (3) the evidence of digestion of mycelium by mesophyll cells (see footnote 1). The association of *Calluna* and other ericaceous species with soils deficient in nitrates in itself provides *raison d'être* for the remarkable biological relations between plant and fungus, assuming fixation of atmospheric nitrogen on the part of the latter.

Experimental observations

CALLUNA SEEDLINGS IN MEDIA LACKING COMBINED NITROGEN.—In experimental cultures, seedlings of *Calluna* grow readily under aseptic conditions in a dilute normal solution made with 1.2 per cent agar-agar. In order to test the possibility of cultivation in a

[5] RAYNER, M. C., The ecology of *Calluna vulgaris*. New Phytol. **12**:59–77. 1913.

substrate free from combined nitrogen, pure culture seedlings were planted in a similar medium lacking nitrates, both sets of seedlings being infected from a pure culture of the endophyte at planting. These cultures were first grown in 1915, and no special precautions were observed beyond the use of pure chemicals and freshly distilled water. The seedlings not supplied with nitrate grew surprisingly well. They

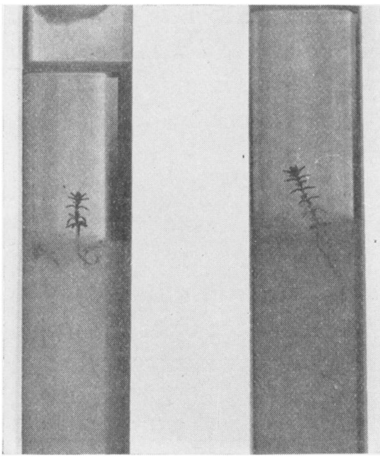

FIG. 3

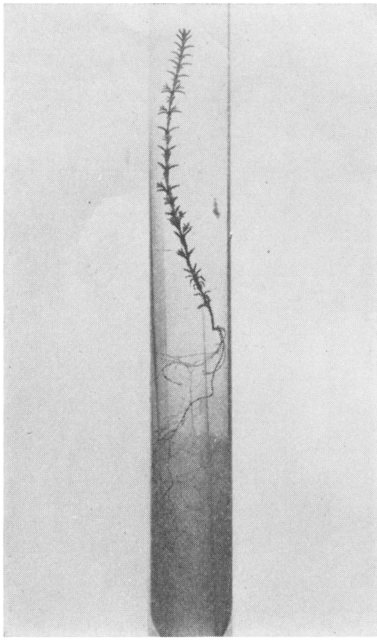

FIG. 4

FIGS. 3, 4.—FIG. 3, control seedlings planted simultaneously with those in fig. 2 in silica jelly nutrient containing combined nitrogen in form of KNO_3; fig. 4, same seedling as shown in right-hand tube of fig. 2, five and a half months old; shoot reached height of over 6 cm.

were, on the average, healthier than the controls, a brighter green, and of quite as vigorous growth. The controls were supplied with potassium nitrate in the proportion of 0.5 gm. per liter. They showed no differentiating features and unfortunately were not photographed. It should perhaps be mentioned that the cultures were grown in a small cold greenhouse away from the laboratory. Kjeldahl estimations of samples of the agar medium subsequently made yielded negative results.

It had been observed previously that *Calluna* seedlings thrive only in solutions of extremely low total concentrations of salts (0.05 per cent). The experiments just described show further that in cultures of "synthetic" seedlings, the use of a culture fluid of 0.05 per cent total concentration affords optimum conditions for growth.

The experiments have since been repeated, using every possible precaution to avoid contamination by traces of combined nitrogen. A similar solution of inorganic salts was made up in silica jelly prepared from specially purified materials and ammonia-free water. The cultures were planted in the autumn under unfavorable weather conditions, and seedlings did not root freely in the silica jelly, which seemed to offer mechanical difficulties. Otherwise, the results confirmed those already described. The seedlings deprived of combined nitrogen were green and healthy and grew at the same rate as the controls.

It may be objected that seedlings of *Calluna* could grow for several months on the seed reserves, and that this accounts for the vigor and longevity shown by seedlings supplied with distilled water only. Against this interpretation is the fact that seedlings germinated on moist filter paper from sterilized seeds not only form no roots, but make practically no shoot growth and quickly show symptoms of starvation such as yellowing and discoloration of the leaves. These symptoms are relieved by inoculation from a pure culture of the endophyte. Finally, there can be no doubt that the optimum conditions in artificial cultures for the establishment and maintenance of a properly balanced relation between plant and endophyte are supplied by a rooting medium of extremely low concentration of salts (for example, 0.05 per cent) lacking combined nitrogen. A fresh line of research is hereby suggested in order to ascertain whether the unfavorable symptoms shown by seedlings planted in culture solutions of higher concentration of salts can be specially correlated with the supply of nitrates. It is certain that a very small alteration in the character of the nutrient supplied to "synthetic" seedlings overthrows the normal balance and induces parasitism in the endophyte.

(3) Additional evidence on nitrogen fixation in Phoma.—An indirect contribution to the subject has recently been made by

DUGGAR (*loc. cit.*). In the course of an experimental review of previous work on nitrogen fixation by fungi, DUGGAR has repeated and extended the observations of earlier workers on this subject, taking extraordinary precautions to avoid experimental methods open to criticism on the score of inaccuracy. Among the species thus investigated are *Penicillium* spp., *Aspergillus niger*, *Macrosporium commune*, *Glomerella Gossypii*, and *Phoma Betae*, as well as three forms of *Azotobacter* isolated from different soils. With regard to the four first named genera, DUGGAR'S work confirms that of previous observers, namely, that these fungi can utilize atmospheric nitrogen to a very slight extent. The amounts recorded are very small, and in DUGGAR'S opinion cannot be accepted as conclusive evidence of ability to fix atmospheric nitrogen. On the other hand, the values obtained by DUGGAR for *Phoma Betae* range from 3.022 mg. to 7.752 mg. per 50 cc. of culture fluid, a known amount of combined nitrogen being supplied. These values are of special interest for comparison with those recorded by TERNETZ for the forms of *Phoma radiciis* extracted from the roots of ericaceous species. Indeed, the evidence appears to be conclusive that ability to continue to fix atmospheric nitrogen exists in varying degree in *Phoma*.

The experimental results obtained by the writer indirectly support this view, and provide a basis for an intelligible explanation of one physiological aspect of the relation between green plant and fungal symbiont in Ericaceae; incidentally, they throw light on the proved ability of species such as *Calluna* and *Vaccinium* to thrive in soils deficient in nitrates. The degree of nitrogen fixation by the endophyte doubtless varies with the species concerned, and may operate as an important survival factor for the plant growing under competitive conditions.

Summary

1. In 1907 TERNETZ provided evidence that certain strains of *Phoma*, isolated from the roots of ericaceous plants, could utilize atmospheric nitrogen.

2. In 1915 the necessary proof that the fungi extracted by TERNETZ were actually the endophytes was provided by the

writer, who showed also that seedlings of *Calluna vulgaris* in pure culture thrive in a rooting medium lacking combined nitrogen.

3. In 1916 DUGGAR offered additional evidence for fixation of nitrogen by members of *Phoma*.

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